The structure of Panulirus intteruptus hemocyanin propbed with moclonal antibodies.

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Summary

This thesis describes the interactions of a number of monoclonal antibodies with hemocyanin from *Panulirus interruptus*. Hemocyanins are a group of copper-containing proteins that are able to bind oxygen reversibly. These proteins play a role in the oxygen transport of arthropods and molluscs, but do not occur in vertebrates. A colorless hemocyanin solution will become blue upon oxygenation.

Hemocyanins have been the subject of many studies in the Department of Chemistry of the Rijks-universiteit Groningen. In the electron microscopy group of van Bruggen the work was started with studies of the higher order structures of a large number of arthropodan and molluscan hemocyanins. In the group of Drenth-Hol work was done on hemocyanins from two arthropods; they solved the X-ray structure of hemocyanin from the spiny lobster *Panulirus interruptus*. Work on *Limulus polyphemus* hemocyanin resulted in some interesting findings about the influence of oxygen binding on the structure of this protein. The group of Beintema solved the primary structure of all subunits of *Panulirus interruptus* hemocyanin and of one subunit of its close relative *Palinurus vulgaris*. In arthropods hemocyanin is present as hexamers or multihexamers built from generally different subunits, that bind oxygen cooperatively.

Injection of a mammal with a protein will result in a defense reaction against it. This protein, the antigen, will be recognized by the products of this reaction; the antibodies. Binding of an antibody to an antigen labels it for further processing by specialized cells of the immune system. The phenomenon of a protein being recognized by antibodies is called antigenicity. It turned out that not each protein or each part of an protein is antigenic to the same extent. The antigenicity of a protein is dependent upon the amino acid sequence and the folding of the protein.

The primary structures of many proteins that are part of pathogens as for instance viruses become available by current molecular biological techniques. In principle it is possible to use only parts of these proteins to raise an immune response against the pathogens. For this information about the antigenicity of these parts is necessary. As already mentioned the primary, secondary and tertiary structure of *Panulirus interruptus* hemocyanin have been solved. The combination of high antigenicity and wealth of available structural information makes the hemocyanin of *Panulirus interruptus* hemocyanin a good candidate to study the relation between antigenicity and structure.

An introductory chapter about the backgrounds of the research is followed in chapter 2 by the description of the production of 18 monoclonal antibodies (IgG-A - IgG-P, IgG-T en IgG-W) recognizing the *Panulirus interruptus* hemocyanin by fusion of a mortal antibody

producing cell with an immortal myeloma cell. The number of obtained monoclonal antibodies as a result of three fusions was rather small, but sufficient for our studies. Each antibody was further characterized. All light chains belong to the κ-type and the heavy chains to the subclasses IgG-1 and IgG-2a. The dissociation constants of complexes of antibodies with antigen varied between 10^{-7} and 10^{-12} M.

The specificities of the antibodies were subsequently determined in chapter 3. Is an antibody able to recognize a peptide fragment of the antigen, or destroys partial or complete denaturation the ability of the antibody to recognize the antigen completely? It turned out that antigenicity of most of the antibodies was not dependent upon the intactness of hemocyanin: the apoprotein was recognized as good as the intact protein. Complete denaturation however, resulted in loss of antigenicity for all antibodies with the exception of IgG-T. All other antibodies do recognize epitopes that are dependent upon folding: they recognize conformational epitopes. Denaturation of the antigen in solution followed by partial renaturation resulted in diminished affinity in about half the antibodies. Most of the other ones recognized the renatured protein which resembles the native protein, as good as the original antigen.

The three dimensional structure of a subunit of *Panulirus interruptus* hemocyanin can be divided into three domains. Cleavage of the protein under mild conditions results into two major fragments representing about 95% of the protein. The conformation of these fragments resemble that of the intact protein. The N-terminal domain is one fragment, the other fragment consists of the two C-terminal domains. Binding of the different antibodies to these fragments resulted in information about the position of the epitopes. The reaction with hemocyanins of different species indicated that most of the monoclonal antibodies showed no or neglible cross-reactivity with hemocyanins of the other crustaceans. The antibodies that showed some reaction with other species showed generally a lower affinity for these hemocyanins as their primary structure differed more with that from *Panulirus interruptus* hemocyanin. IgG-T surprisingly showed a comparable or even higher affinity for *Palinurus vulgaris* hemocyanin as for that of *Panulirus interruptus*. This observation together with a strong binding with a C-terminal peptide fragment resulted in the elucidation of the sequential epitope of IgG-T on the primary structure of *Panulirus interruptus* hemocyanin. Dimerization of this peptide leads to an increase in binding affinity (chapter 4).

In chapter 5 some methods to map the conformational epitopes by studying the formation of ternary complexes are described. A ternary complex consists of two antibodies or their fragments that bind simultaneously to a monomeric hemocyanin molecule. A competitive-binding immunoassay method was more successful in the determination of ternary complexes than gel electrophoresis and gel filtration. The latter two methods could only be applied with antibodies possessing a high-affinity. The epitopes of eleven monoclonal antibodies were assigned to five groups on the basis of these interactions. By combining these results with those of chapters 2 and 3, three of the epitopes are placed on the first domain and two on domains 2 and 3.

A more precise method to localize the epitopes uses electron microscopy of antibody-hemocyanin complexes in combination with image processing of a large number of complexes (chapter 6). On a flat surface *Panulirus interruptus* hemocyanin gives two views: one

perpendicular to the threefold axes, a rectangular view, and one parallel to the threefold axes of the hexamer, an hexagonal view. These views can also be simulated by using the known three-dimensional structure of the protein. The simulation clarifies parts of the amino acid sequence which can be located in the electron microscopy pictures. By comparison of the averaged images of antibody-hemocyanin complexes of IgG-E and IgG-J with simulated images of complexes, the position of the epitopes within a subunit could be localized for these two antibodies. The results confirmed those of chapter 5. Although this method is not good enough to pinpoint the epitope on a few amino acids the conclusion can be drawn that the epitopes are located roughly on several loops containing a relative high percentage of charged amino acid residues.

How the binding of three antibodies IgG-D, IgG-E and IgG-J effect the oxygen binding behavior of monomeric and hexameric hemocyanin of *Panulirus interruptus* hemocyanin is studied in chapter 7. The influence of the intact antibodies and their antigen binding fragments (Fab) have been investigated. Two antibodies increase the oxygen affinity of monomeric hemocyanin from that observed in its low-affinity state, while the third one has little influence on this property. Binding of antibody fragments abolishes almost completely the cooperativity of oxygen binding by the hexameric hemocyanin molecule. The two antibodies which increase the oxygen affinity of the monomeric molecule stabilize high-affinity states of the hexameric molecule, while the third one stabilizes the low-affinity state. These antibodies react with other surface parts of the subunit than the second domain in which the oxygen binding site is located. Apparently there are conformational changes in the other domains which influence oxygen affinity in the second domain. Therefore they can play a role in the regulation of the oxygen affinty of *Panulirus interruptus* hemocyanin.